Structure and Function of Metal- and Nitrate-reducing Microbial Communities in the FRC Subsurface

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Abstract

The overall goal of this study is to evaluate structure-function relationships of sedimentary microbial communities likely to regulate U(VI) reduction and immobilization in the subsurface of Area 2 at the Field Research Center (FRC), Oak Ridge, TN. Microcosm experiments were conducted under near *in situ* conditions with FRC subsurface materials cocontaminated with high levels of U(VI) and nitrate. The activity, abundance, and community composition of microorganisms was determined in microcosm samples, stimulated with ethanol or glucose, and compared to those from sediment cores and unamended controls. Activity was assessed by monitoring terminal electron accepting processes (TEAPs; nitrate, sulfate, uranium, and iron reduction) as well as electron donor utilization. Microbial functional groups, nitrate- and iron(III)-reducing bacteria, were enumerated during the nitrate- and metal-reduction phases of the incubation and in sediment core samples using a most probable number (MPN) serial dilution assay. U(VI) and Fe(III) were reduced concurrently in the glucose but not the ethanol treatments. In ethanol-amended microcosms, U(VI) was reduced during a 4-day lag phase between nitrate- and Fe(III)-reduction phases. Biostimulation resulted in 3 to 5 orders of magnitude higher counts of Fe(III)-reducing bacteria, whereas populations of nitrate-reducers were enhanced by 1 to 3 orders of magnitude. One to 2 orders of magnitude more Fe(III)-reducers were observed in ethanol- as compared to glucose-amended treatments in parallel with enhanced U(VI) removal in ethanol treatments. Cultivatable Fe(III)-reducing bacteria in the ethanol treatments were dominated by *Geobacter* sp. while those cultured on glucose were dominated by fermentative organisms, i.e., *Tolumonas* sp. Currently, carbon substrate utilization is being examined through HPLC analysis of microcosm porewaters. In addition, changes in the overall microbial community composition are being assessed using cultivation-independent techniques, including fluorescence *in situ* hybridization (FISH), terminal restriction fragment length polymorphism analysis (T-RFLP) and cloning/ sequencing of structural and functional genes. Our results indicate that the microbially-catalyzed mechanism of U(VI) reduction is electron donor dependent and that more effective U(VI) removal is achieved in parallel with an enrichment of *Geobacter* sp. upon treatment with ethanol.

Hypotheses/ Objectives

- **We hypothesize that U(VI) remediation potential is dictated by the physiological requirements for the growth and metabolism of subsurface microorganisms.**
- **Therefore, we examined the coupling between the function or activity of sediment-associated microbial communities and community composition under near in situ conditions in microcosms of subsurface materials.**

Experimental Approach

Microcosms

- **Area 2 sediment (FB094) was combined with Area 2 groundwater (FW209).**
- **Microcosms were sealed, neutralized and flushed with N_2.**
- **Treatments (3 replicates each):**
  - **20 mM Ethanol**
  - **10mM Glucose**
  - **Unamended control**
- **Incubated at 30°C and sampled for geochemical analysis (nitrate, Fe(II), & U(VI)) every 1-5 days)**

Microbial Community Characterization

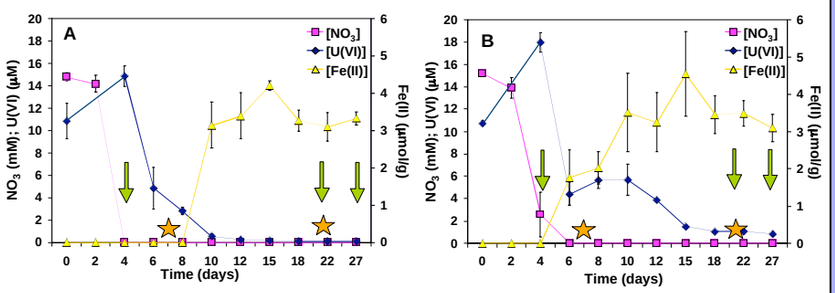
Cultivation-Dependent Community Analysis

- **A most probable number (MPN) dilution series was used to enumerate nitrate- and iron-reducing bacteria present in the microcosms (Fig. 1) and FB094 sediment.**
- **After 3 months of growth the highest positive dilutions were sampled for NO_3^- and Fe(III)-reduction, HPLC and molecular analysis (SSU rRNA).**

Cultivation-Independent Community Analysis

- **DNA was extracted from microcosm samples (Fig. 1) and MPN cultures.**
- **Two approaches were used to characterize microbial communities:**
  - **1) Cloning and sequencing of PCR amplified SSU rRNA genes.**
  - **2) Fingerprinting using terminal restriction fragment length polymorphism (TRFLP).**

Figure 1: Electron acceptor usage in (A) ethanol and (B) glucose amended microcosms. Arrows indicate sampling points for TRFLP community fingerprinting. Stars indicate sampling points for inoculating MPN dilution series.



Results: Enumeration and Identification of Fe(III)- and NO_3-reducing Bacteria

Table 1: Enumeration of Fe- and NO_3-reducing microorganisms in microcosm sediments. Abundance (cells/ml) determined using an MPN serial dilution assay.

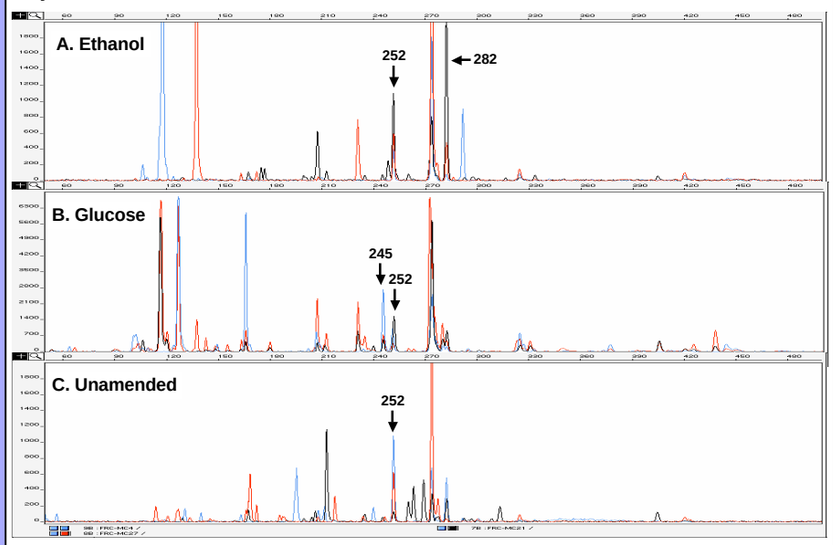
Incubation Time	Treatment	Carbon Substrate	Electron Acceptor	
			FeOOH	Nitrate
Day 7 (Nitrate reduction phase)	Glucose	Glucose	10^6	10^9
	Ethanol	Ethanol	10^7	10^8
Day 21 (Iron reduction phase)	Glucose	Glucose	10^7	n/a
	Ethanol	Ethanol	10^7	10^8
FB094 Sediment	Unamended	Glucose	10^2	10^6
	Unamended	Ethanol	10^2	10^7
	Sediment	Glucose	10^3	10^7
	Sediment	Ethanol	10^4	10^4

Characterization of Fe(III)-reducing bacteria from day 21:

- **Clones from the glucose treatment were related to *Tolumonas* sp. and *Clostridium* sp.**
- **Bacteria cultivated in the ethanol treatment were related to *Geobacter metallireducens***

Results: TRFLP Community Fingerprinting of Microcosm Sediments

Figure 2: SSU rRNA TRFLP profiles of (A) ethanol, (B) glucose and (C) unamended microcosm treatments. Profiles corresponding to day 4 of the incubation are in blue, day 21 in black and day 27 in red.



Analysis of TRFLP Profiles

- **Peak Identification**
  - **SSU rRNA sequences derived from studies at the FRC site were compiled.**
  - **Sequences containing the 27F forward primer were digested *in silico* with *Mnl*I.**
  - **Peaks from TRFLP profiles were compared to results from *in silico* digests:**
    - **35-60% of peaks were identified**
    - **40-82% of the total peak area was identified**
- **Peaks of Interest:**
  - **252 bp = detected in all treatments; related to *Alphaproteobacteria* (*Hyphomicrobiaceae*)**
  - **282 bp = detected only in ethanol and unamended treatments; related to *Arthrobacter* spp. member of the *Actinobacteria* (*Micrococcaceae*)**
  - **245 bp = detected only in glucose treatment; related to *Clavibacter* spp. member of the *Actinobacteria* (Microbacteriaceae)**

Results: Community Characterization of Microcosm Sediments

Figure 3: Frequency of bacterial phylogenetic lineages detected in the SSU rRNA clone library constructed from pooled DNA extracts of microcosm sediments. Calculations based on the total number of clones associated with a sequenced phylotype.

- **74 clones; 10 phylotypes**
- **Phylogenetic groups detected capable of nitrate reduction, metal reduction and fermentation.**
- ***Betaproteobacteria***
  - ***Alcaligenes defragrans, Diaphorobacter* sp., & *Dechloromonas* sp.**
- ***Gammaproteobacteria***
  - ***Tolumonas* sp.**
- ***Firmicutes***
  - ***Clostridium* sp.**

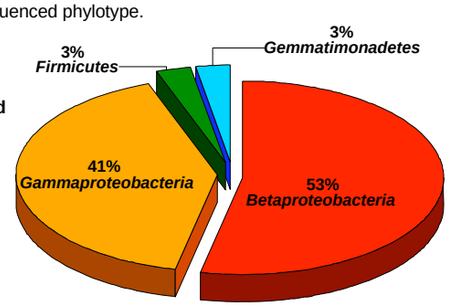
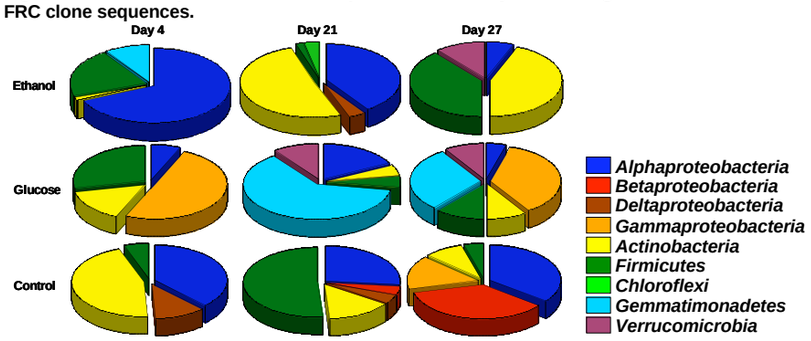


Figure 4: Percent of peak area of TRFLP peaks identified by *in silico* digests with *Mnl*I of FRC clone sequences.



Conclusions

- **Terminal-electron-accepting processes were electron donor specific**
  - **Nitrate and metal reduction occurred with a minimal lag phase in the glucose treatments, whereas in ethanol amended treatments a 4-day lag phase was observed.**
  - **Ethanol treatments were also shown to more effectively reduce U(VI).**
- **Electron flow in microcosms is defined by fermentative metabolism and incomplete oxidation of ethanol.**
- **The impact of nitrate on the abundance of Fe(III)-reducers was clearly observed in the MPNs of control treatments in comparison to sediment core samples.**
- **The polyphasic approach utilized in this study revealed microbial communities capable of both nitrate and metal reduction.**
  - **Nitrate-reducers included *Hyphomicrobium*, *Alcaligenes*, *Diaphorobacter* & *Dechloromonas* groups.**
  - **Fe(III)-reducers included *Geobacter* and *Clostridium.***
- **Cultivatable Fe(III)-reducers were dominated by respiratory and fermentative organisms in ethanol and glucose treatments, respectively.**
- **The relative abundance of microbial groups from TRFLP analysis was substantially different from clone library analysis.**
- **Analysis of TRFLP profiles for Area 2 microcosms revealed changes in the phylogenetic groups detected in samples from all treatments with incubation time.**
- **High throughput extraction followed by TRFLP analysis provides a rapid, inexpensive method for screening of sediment-associated microbial communities.**
- **Independent verification of clone library analysis is warranted and should become standard practice.**

Future Work

- **Screen additional clones from microcosm clone libraries.**
- **Verify *in silico* digests with TRFLP profiles by fingerprinting clones.**
- **Characterize the cultivated nitrate-reducing bacteria derived from the microcosms.**
- **Utilize quantitative methods (MPN-PCR; real-time PCR; FISH) to verify and support the semi-quantitative interpretation of TRFLP profiles.**

Acknowledgments
This research was funded by ERSP/ OBER, U.S. DOE (grant DE-FG02-00ER62986). We thank David Watson, Tony Palumbo, and Lisa Fagan for sediment sampling and collaboration. Andrew Madden provided the U(VI) analyses through a collaboration with ORNL. We would like to thank Lora McGuinness and Jon Delgardio for their aid with microbial community analysis.